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Genetic Characterization of Desi Cotton Hybrids Using Molecular Markers

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A B S T R A C T

The desi cotton hybrids *viz*., G.Cot.DH-7 (Sujay x G 27) and G.Cot.DH-9 (4011 x 824) and their parents were characterized using morphological and molecular markers. The desi cotton hybrids and their parents showed variations for morphological characters *viz*., foliage, leaf shape, leaf hairiness, growth habit, pollen colour, bolls shape and stem hairiness. Based on the distinct morphological characters one can identify true hybrid. A set of 25 RAPD and 19 ISSR were used for molecular characterization of desi hybrids. Primer ISSR9 amplified two polymorphic loci designated as ISSR9_200bp (Sujay) and ISSR9_550bp (G.27) in G.Cot.DH-7; this heteroallelic marker not only differentiated the parents but also confirmed true hybrid. RPI7 amplified a polymorphic marker of approximately 450 bp and 500 bp in the female parent (4011) and hybrid G.Cot.DH-9 but not in the male parent (824). Similarly, RPI8 primer amplified allele of 550bp in the male parent and the hybrid G.Cot.DH-9, but not in its female parent. Thus, the combination of RPI7_450 (4011) and RPI8_550 (824) marker can be used for identification of G.Cot.DH-9. Our study revealed the parentage and desi hybrid confirmation using molecular marker and the markers identified would be valuable genomic tool for desi cotton breeding programs.

Introduction

Cotton is one of the most important commercial crops popularly known as the "White Gold". The existence of cotton thread in India is traced back to the *Rigveda* about 4000 B.C. and India is not only the birth place of cotton but also only country to grow all four species of cultivated cotton on commercial scale, two tetraploid called new world cotton (*Gossypium hirsutum and Gossypium barbadense*) and two diploid called old world cotton or *desi* cotton (*Gossypium herbaceum* and *Gossypium arboreum*). India also has a pride place in the global cotton scenario due to several distinct features such as the largest cotton growing area, cultivation of all the four cultivated species, large area under tetraploid cotton, one of the largest producers of long and extra-long staple cotton, native home of old world cultivated cotton and wide diversity in agro-

climatic conditions under which cotton is grown. During the pre-independence era, about 97 per cent of cotton area was under Asiatic (*desi*) cottons and only 3 per cent area was under American cotton (*G. hirsutum*). Asiatic (*desi*) cottons are known for their good agronomic base, strong resistance to disease and pest, abiotic stress tolerance (salinity and drought) and suitability under rainfed conditions (Patel and Chaudhari, 2015). After the introduction of *Bt* cotton, area under cultivation of *desi* cotton decreased significantly but now there is a big demand of short staple cotton for denim and surgical cotton, and hence price of short staple cotton is increased due to shortage of *desi* cotton (Patel and Chaudhari, 2015).

The varieties and hybrids attain acceptance when the farmer gets genetically pure seeds of high standards. For this purpose, each cultivar should be properly defined with suitable descriptors, so as to maintain its identify during seed production through field inspection and certification. Apart from this, characterization of cultivars is also required for their protection under PPV & FR Act 2001. In India, while certain diagnostic features for released and notified crop varieties and hybrids are known and used in seed certification, but by and large descriptors are incomplete. Traditionally, it has been the practice to carry out Grow Out Test (GOT) to analyze the genetic purity of hybrid seeds using morphological traits (Ankaiah *et al.*, 2005; Tatineti *et al.*, 1996;). However, morphological differences between true hybrids and off types are not always apparent and cannot be recognized easily, especially when the parents are genetically similar. The sensitivity of morphological traits to the environment further limits the application of GOT. With the advent of the molecular marker technology, it is now possible to test the purity of the hybrid seed immediately after harvesting and processing by DNA markers. DNA markers such as RFLP (Pendse *et al.*, 2001; Dongre and Parkhi, 2005) RAPD (Venu, 2001; Rao *et al.*, 2002; Mehetre *et al.*, 2007), AFLP (Rana and Bhat, 2004), SSR (Rana, 2003; Dongre and Parkhi, 2005; Saravanan *et al.*, 2007) and ISSR (Dongre and Parkhi, 2005; Rana *et al.*, 2006) have been used to rapidly screen genetic purity of hybrids. The objective of the present study was to test genetic purity and identify the markers for desi cotton F1 hybrids G.Cot.DH-7 and G.Cot.DH-9 and its parents using RAPD and ISSR markers.

Materials and Methods

The material for the present investigation comprised of *intraspecific* cotton F_1 hybrids *viz*., G. Cot.DH-7 its parents Sujay (female) and G-27 (male); and G. Cot. DH-9 and its parents 4011 (female) and 824 (male) were obtained from Main Cotton Research Station (MCRS), Navsari Agricultural University (NAU), Athwa Farm, Surat.

Grow out test (GOT) has been employed to assess the purity of hybrid seeds using morphological traits. The traits such as foliage, leaf shape, lobs, leaf hairiness, growth habit, pollen colour, bolls shape, stem hairiness, and other DUS testing character were studied to distinguish the hybrids from off types.

DNA isolation

Four to five fresh, young, healthy leaves were collected from the hybrids G.Cot.DH-7 and G.Cot.DH-9 along with their parents and kept at 4ºC in the sterilized cooling box to avoid drying and minimize the phenolics compound level and brought to laboratory for DNA isolation. Genomic DNA isolated from young fully expanded leaves of hybrids and their respective parents using the CTAB method with minor modification (Saghai-Maroof *et*

al., 1984). Quality and quantity of DNA were estimated by measuring O.D. at 260/280nm in UV Spectrophotometer. Intactness of genomic DNA was checked on agarose gel (0.8%).

RAPD and ISSR amplification

The genomic DNA is subjected to PCR. A total of 25 RAPD and (GeNei primers: RPI1 to RPI25) and 19 ISSR (ISSR 1 to ISSR 15) primers were used for hybrid analysis. RAPD and ISSR each of 25µl reaction volume were carried out in 200µl PCR tube. Each reaction mixture (25µl) for PCR amplification consisted of 10 X reaction buffer (10 mM Tris-HCl, pH 8.3 and 50mM KCl), 2.5 mM $MgCl_2$, 3U Taq DNA polymerase; 2.5 mM each dATP, dTTP, dCTP and dGTP (all reagents from Fermentas), 10 pico mole primer (Bangalore GeNei India), and 10 ng genomic DNA template.

The DNA amplification was carried out in thermocycler. For RAPD analysis, an initial denaturing step of 6 min at 94˚ C was followed by 35 PCR cycles (denaturing at 94˚ C for 45 sec, primer annealing at 36˚ C for 1 min and primer extention at 72˚ C for 2 min). For ISSR analysis, an initial denaturing step of 10 min at 94˚ C was followed by 35 PCR cycles (denaturing at 94˚ C for 1 min, primer annealing at 49˚ C for 1 min and primer extension at 72˚ C for 2 min). A final step of 10 min at 72˚ C was carried out for polishing the ends of PCR products in all RAPD and ISSR markers.

Resolution of PCR products

Amplified DNA fragments were separated on 1.5 % agarose gels and stained with ethidium bromide. Running buffer containing TBE [Tris-buffer, boric acid and EDTA (pH 8.0)] was used for electrophoresis. Wells were loaded with 15µl of amplified sample and 3µl of loading buffer (sucrose, bromophenol blue and Xylene cyanol). The gel was run at 80 V (constant) to separate the amplified bands. The standard DNA marker (100 bp) was also run along with the samples. The separated bands were documented under UV transilluminator and photographed by Gel documentation system (Alpha Innotech. U.S.A.) and analyzed.

Results and Discussion

Morphological characterization

The hybrid G.Cot.DH-7, G.27, and 4011 exhibited erect type of growth habit, while G.Cot.DH-9, Sujay, and 824 had semi-erect type of growth (Table 1). The variation exhibited by the genotypes for growth habit is due to genetic character of the genotypes. The genotypes exhibited variation for stem hairiness and grouped as sparsely hairy (G.Cot.DH-7, G.Cot.DH-9, 4011, and 824) and glabrous (Sujay). Similarly, Sethi *et al.*, (1960) reported that stem hairiness is a genetically controlled and it varies from genotype to genotype. However, the five lobs per leaves were observed in both hybrids and their parents. The leaf foliage colour of G.Cot.DH-7 and Sujay was light green and G.27 was dark green. Whereas in case of hybrid G.Cot.DH-9 and female parent 4011 leaf were green and male parent (824) was dark green in colour. The genotypic variation in the colour of the leaf may be due to genetic character of the parents and may be also due to edaphic and environmental conditions such as nutritional factors and light intensity during crop growth.

All the genotypes except 824 have medium hairs on leaves. However, the earlier study revealed that hair on leaf behaves like a simple mendelian trait giving 1:2:1 ratio in crosses between sparsely hairy and medium hairy leaves (Harland, 1944). The petal colour of the flower is one of the important

characters for characterization, based petal color the genotypes were categorized as cream (G.27), yellow (G.Cot.DH-9, G.Cot.DH-7, Sujay and 824) and deep yellow (4011). Similarly, petal spot is used as marker for varietal identification, which is present in G.Cot.DH-9, G.Cot.DH-7, Sujay and 824 and absent in 4011 parent. The variation in petal spot is due to genetic constituent of the genotypes which has cumulative effect and is governed by dominant gene as it was reported by Ahuja *et al.*, (2006) in cotton. The variation in the anther colour was noticed and the genotypes were grouped as dark yellow in both the female parent (Sujay and 4011) as well as hybrids (G.Cot.DH-7 and G.Cot.DH-9) and light yellow in male parents G.27 and 824, which is a heritable character and it is genetically controlled. The cylindrical boll shape observed both the hybrid and their parent except 824 (elongated) and it is genetically controlled by the genotype.

Molecular characterization

The assessment of genetic purity of hybrid using GOT has limitations, warrants faster, reliable and precise assessment of genetic make up of a genotype through molecular markers. Thus, in the present study aimed at confirmation of cotton F1 desi hybrids *viz*., G.Cot.DH-7 (Sujay × G.27) and G.Cot.DH-9 (4011 × 824) using 25 RAPD and 19 ISSR primers.

Identification the hybrid G.Cot.DH-9

Out of 25 RAPD, two decamer random primers RPI-7 and RPI-8 were found extremely polymorphic between male and female parents respectively. These highly informative primers not only differentiated 4011 and 824 but also confirmed the parentage of their G.Cot.DH-9 hybrid. The two random primers amplified collectively six fragments, while the number of resolvable loci was two and one for the primers RPI-7and RPI-8, respectively. RPI-7 generated a polymorphic marker of approximately 450bp and 500bp in the female parent (4011) and in hybrid but not in the male parent. However, the RPI-8 primer amplified a different allele of size 550bp in the male parent (824). The same allele size of 550bp was expressed in the hybrid, but not in its female parent. The presence of both female and male parent alleles indicated the result of crossing between two parents (hybrid). The observed banding pattern was highly specific to this hybrid (Figure 1). Polymorphic molecular markers produced unique banding and not only discriminated the two cotton parents, but also identified their true hybrids. Polymorphism revealed by RAPDs is based on the position and orientation of primers annealing site and the interval they span. Polymorphism between individuals can arise through nucleotide substitutions and insertions or deletions (Williams *et al*., 1990). This technique can be adopted for large scale screening of hybrids in cotton (Dongre and Parkhi 2005).

Identification the hybrid G.Cot.DH-7

Among 25 RAPD primers, RPI-7 identified two female specific allele amplicons about 450bp and 500bp for hybrid G.Cot.DH-7. Further, out of 19 ISSR primers, two primers ISSR-9 and ISSR-12 showed polymorphism in parents. Out of which only one primer ISSR-9 was found to be heteroallelic for parents. ISSR-9 produced two distinguishable amplicons, amplicons about 200bp was specific to female (Sujay) and another amplicons about 550bp was specific to male parent (G.27) (Figure 2). Whereas, ISSR-12 primer resulted in amplification of 700bp male specific amplicon that was present in hybrid G.Cot.DH-7.

Table.1 Morphological characters of cotton hybrid and its parents

Sr. No.	Character	Female (Sujay)	Male (G.27)	G.Cot.DH-7	Female (4011)	Male (824)	G.Cot.DH-9
1	Foliage	Light Green	Dark Green	Light green	Green	Dark Green	Green
2	Leaf shape	Semidigitate	Palmate	Semidigitate	Palmate	Palmate	Palmate
3	Lobs	5 lobs	5 lobs	5 lobs	5 lobs	5 Lobs	5 Lobs
4	Leaf hairiness	Medium	Medium	Medium	Medium	Sparse	Medium
5	Habit	Semi-erect	Erect	Erect	Erect	Semi-erect	Semi-erect
6	Pollen	Dark yellow	Light yellow	Dark Yellow	Dark yellow	Light yellow	Dark yellow
7	Red petal spot	Present	Present	Present	Absent	Present	Present
8	Petal colour	Yellow	Cream	Yellow	Deep yellow	Yellow	Yellow
9	Bolls shape	Cylindrical	Cylindrical	Cylindrical	Cylindrical	Elongated	Cylindrical
10	Stem hairiness	Glabrous	Sparsely Hairy	Sparsely Hairy	Sparsely Hairy	Sparsely Hairy	Sparsely Hairy

Figure.1 Amplification profile of cotton parents and their F1 with polymorphic RAPD primers (RPI-7 and RPI-8) M: marker (DNA ladder), F: female parent one (4011), DH-9: first generation hybrid (G.Cot.DH-9), M: male parent (824)

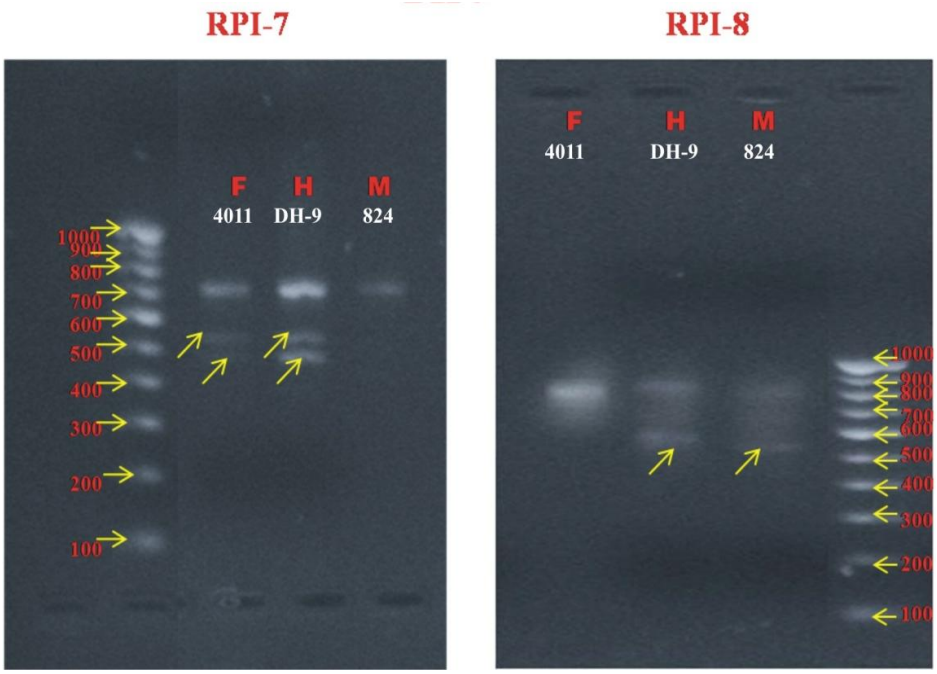
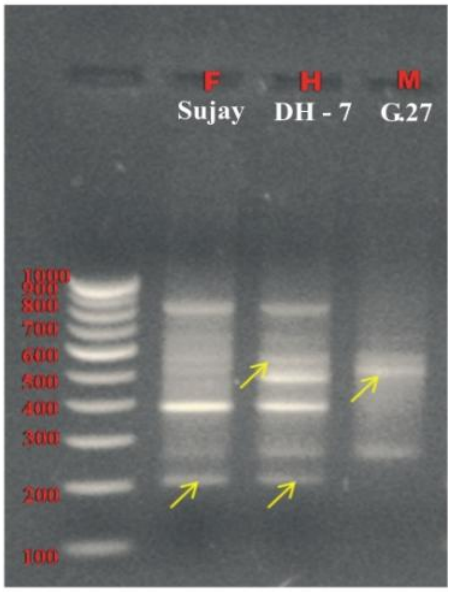


Figure 2 Amplification profile of cotton parents and their F1 with polymorphic ISSR 9. [M: marker (DNA ladder), F: female parent one (Sujay), DH-7: first generation hybrid (G.Cot.DH-7), M: male parent (G.27)]



Our results confirm that ISSR markers are efficient tools for the discrimination of F_1 hybrids from the self-pollinated progeny of female parents in controlled crosses. They can also be effectively used to fingerprint and differentiate plants with highly similar morphological characteristics. The hybridity status of F1 hybrids can be easily verified by comparing amplified polymorphic bands between F1 hybrids and parents (bands specific to the female and male parent). In addition, ISSR markers allow the easy, fast, inexpensive, accurate, reliable, and simultaneous detection of polymorphisms at multiple loci in the genome using low quantities of DNA. These properties have made the markers useful for the genetic analysis of various plants (Reddy *et al.*, 2002). Our results agreed with those of Desai (2014), who reported the use of 19 ISSR primers to identify the parentage of hybrids of cotton. Similarly, Liu *et al*., (2007) have used 54 ISSR primers to confirm the hybrids of three crosses of the cotton and found that two ISSR primers gave polymorphic DNA bands between male and female parents in each cross and could verify their interspecific hybrids. Eight ISSR primers have also been used successfully in the identification of cotton hybrids (Dongre *et al*., 2005, 2010 and 2012).

Microsatellite repeats used as primers in ISSR markers, amplify DNA segment present at an amplifiable distance in between two identical microsatellite repeat relatively more stable and repeatable. The polymorphic ISSR markers produced female specific amplicon. Thus ISSRs can be used to identify female parent of the respective hybrid. Though the usefulness and effectiveness of different type of molecular markers were reported, the selection of marker type is of great importance for successful genetic purity testing. In case of dominant markers such as RAPD and ISSR, only those primers which amplify bands specific to the male parent might reveal a proper pattern of a true hybrid as opposed to that of the female parent.

It can be concluded from this study that it is possible to differentiate the desi cotton hybrids more accurately and efficiently from its parental lines using RAPD and ISSR markers. These molecular markers would be more efficient than GOT as markers are independent of environmental influence. Thus, DNA markers would be more accurate for determining hybrid seed purity.

Conflicts of Interest: The authors declare no competing/conflict or financial interests.

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